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Analysis of protein tyrosine phosphatase interactions with microarrayed phosphopeptide substrates using imaging mass spectrometry

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Abstract

Microarrays of peptide and recombinant protein libraries are routinely used for high-throughput studies of protein-protein interactions and enzymatic activities. Imaging mass spectrometry (IMS) is currently applied as a method to localize analytes on thin tissue sections and other surfaces. Here, we have applied IMS as a label-free means to analyze protein-peptide interactions in a microarray-based phosphatase assay. This IMS strategy visualizes the entire microarray in one composite image by collecting a pre-defined raster of MALDI-TOF MS spectra over the surface of the chip. Examining the bacterial tyrosine phosphatase YopH, we used IMS as a label-free means to visualize enzyme binding and activity with a microarrayed phosphopeptide library printed on chips coated with either gold or indium-tin oxide. Further, we demonstrate that microarray-based IMS can be coupled with surface plasmon resonance imaging to add kinetic analyses to measured binding interactions. The method described here is within the capabilities of many modern MALDI-TOF instruments and has general utility for the label-free analysis of microarray assays.

Keywords

MALDI; Imaging mass spectrometry; peptide microarray; YopH; phosphatase assay; SPRi

Introduction

Peptide microarrays are increasingly used in large-scale studies of protein-protein interactions. Short peptide sequences derived from protein motifs are often sufficient to recreate native interactions, and such peptides can be synthesized and printed on a large scale for use in binding or enzymatic assays [1]. Miniaturization of bioassays by high-density printing of microarrays minimizes reagent consumption while maximizing throughput. A significant barrier to implementation of peptide microarrays, however, is the lack of analytical methods to visualize these events. Matrix-assisted laser desorption/ ionization mass spectrometry (MALDI-MS) is a soft ionization technique for analysis of proteins, peptides, lipids, nucleic acids, and other high molecular weight molecules. For MALDI-MS, analytes are co-crystallized with a chemical matrix and ejected into the gas

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phase as ions by laser ablation. MALDI-imaging mass spectrometry (MALDI-IMS) takes advantage of this targeted sampling to acquire data directly from the surface of flat samples, generally thin tissue sections, that have been coated in matrix (reviewed in [2]). This strategy facilitates the identification of analytes with molecular specificity while preserving the original spatial distribution *in situ*.

Phosphatase assays generally employ small chromogenic substrates, pNPP for example, or fluorescently labeled phospho-specific antibodies to visualize phosphorylation status of peptide substrates after exposure to enzyme (reviewed in [3]). False signals in these probe-based assays are influenced by amino acid sequence context, and for the case of antibodies, probes must be specific for a single phosphorylated residue (p-Tyr, p-Ser, p-Thr, etc.). MALDI-IMS can potentially overcome many limitations of probe-based assays by using label-free detection of dephosphorylation based on mass shift. Here, we have applied MALDI-IMS as a novel readout for a peptide microarray-based phosphatase assay. Our experiments measured interactions between the protein tyrosine phosphatase YopH produced by the bacterium *Yersinia pestis* and a small library of phosphorylated peptides derived from a targeted motif [4; 5] in epidermal growth factor receptor (EGFR). Local sequence is thought to play an important role in phosphatase substrate affinity, and the arrayed peptides were designed with substitutions and truncations predicted to affect YopH binding and activity.

Although IMS was previously applied to analyze tissue microarrays and *in situ* synthesized peptide microarrays [6; 7; 8], our study demonstrates that IMS can directly visualize the results of binding and enzymatic assays by providing a detailed characterization of microarrayed substrates at the molecular level. In addition, this method simultaneously evaluates microarray printing quality by localizing and identifying peptides, products and contaminants.

Materials and Methods

Reagents

11-mercaptoundecaonoic acid (11-MUA), α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), formic acid (Fluka brand), and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and N-sulfohydroxysuccinimidyl ester (Sulfo-NHS) were purchased from Thermo Scientific (Rockford,IL, USA).

Peptides and proteins

The following biotinylated peptides were synthesized, purified to ≥95% by HPLC and confirmed by MS by Peptide 2.0 Corp. (Chantilly, VA, USA): Biotin-AHX-VDADA(pY)LI-amide , Biotin-AHX-VAAAA(pY)LI-amide, Biotin-AHX-VDAAE(pY)LI-amide , Biotin-AHX-VAAAE(pY)LI-amide, Biotin-AHX-VAADE(pY)LI-amide, Biotin-AHX-VDADE(pY)LI-amide, Biotin-AHX-VDADEYLI-amide, Biotin-AHX-ADE(pY)L-amide, Biotin-AHX-VVDADE(pY)LIPQQG-amide (Table 1). Peptides were reconstituted for printing (500 μM) in 10mM citrate buffer, pH 6.6. Recombinant *Yersinia pestis* proteins consisting of wild-type YopH (wt-YopH) and catalytically inactivated (mutant C403A/D356A) YopH (m-YopH) were kind gifts of Dave Waugh (NCI-Frederick National Laboratory), prepared as described previously [5]. Mouse anti-pTyr antibody was purchased from Cell Signaling (Danvers, MA, USA, pTyr-100, Cat. # 9411) and NeutrAvidin was purchased from Thermo Scientific (Rockford, IL, USA).

Microarray printing

Microarrays used in these experiments were printed on 25×75×1.1 mm glass slides coated (30-60 ohms) with indium-tin oxide (ITO; Delta Technologies, Loveland, CO, USA) or polycarbonate slides coated with gold (GE Healthcare, Piscataway, NJ, USA). The print surfaces of the slides were covered with self-assembled monolayers (SAM) of 11-MUA by immersion in a 1mM solution in ethanol for two hours (22° C) followed by extensive rinsing with 100% ethanol. Reactive esters were created on the SAM carboxyl moiety by exposure to a solution containing 200 mM EDC and 50 mM Sulfo-NHS in water for ten minutes (22° C). Excess EDC/sulfo-NHS solution was rinsed away with water and a solution of 100 μg/ml NeutrAvidin in phosphate buffered saline (PBS), pH 7.4 was applied to the print surface. Excess NeutrAvidin was rinsed away with PBS followed by water. Unreacted esters were blocked for five minutes with 1M Tris, pH 9.0 (22° C), rinsed with water and dried. The slides were used immediately for microarray printing. Biotinylated peptides were printed on the surface in a 9 × 3 microarray format using an inkjet microarray printer (ArrayJet, Edinburgh, Scotland), delivering 1.5 nl of 500 μM peptide solution to produce spots with1.0 mm center-to-center spacing.

YopH phosphatase and binding assays

For the phosphatase activity assay, 1 μM wt-YopH (amino acid residues 164–468) was prepared in 10 mM citrate buffer, pH 6.6, containing 100 mM NaCl and 1mM DTT. The wt-YopH (200 μl) was applied to the print area of an ITO-coated slide and incubated for 10 minutes (22° C). The protein solution was removed and the print area was washed with 10 mM citrate buffer, pH 6.6, containing 100 mM NaCl, followed by filtered deionized water. The slide was dried thoroughly under a nitrogen gas stream before matrix application. For the binding assay, 2.5 μM of m-YopH was prepared in 10 mM citrate buffer, pH 6.6, containing 100 mM NaCl and 1mM DTT. M-YopH (200 μl) was applied to the print area of an ITO-coated slide and incubated for 30 minutes (22° C). The protein solution was removed and the print area was washed with 10 mM citrate buffer, pH 6.6, containing 100 mM NaCl followed by filtered deionized water. The slide was dried under a nitrogen gas stream before matrix application.

Surface plasmon resonance imaging

SPRi experiments were carried out with a GE Flexchip using a 500 μl/ minute flow rate, temperature of 25° C, and HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20) as a running buffer. SPRi experiments consisted of a 30 minute block step with Flexchip blocking buffer (GE Healthcare), 15 minute equilibration with running buffer, 15 minute injection with anti-phosphotyrosine monoclonal antibody diluted 1:1000 in running buffer supplemented with 1mm dithiothreitol, and two minute dissociation initiated by injection of running buffer. The cover slip of the SPRi microarray chip was carefully removed with tweezers and the microarray surfaces were washed in filtered deionized water and dried under a nitrogen gas stream before matrix application.

Matrix application for MALDI-MS analysis

The MALDI matrices consisted of 5 mg/ml CHCA or 10 mg/ml SA dissolved in 50% acetonitrile aqueous solution with 0.1% formic acid. An automated sprayer (TM-Sprayer, HTX Technologies, Carrboro, NC, USA) was used for matrix application, using 0.25 ml/ minute 50% acetonitrile aqueous solution as a mobile phase, nitrogen pressure of 10 psi (140 °C) as a nebulizing gas and the following settings: 1200 mm/ minute stage velocity, 3 mm track spacing, 1.5 mm offset between consecutive passes, with six total passes (three in horizontal direction and three vertical).

Imaging mass spectrometry

IMS experiments were carried out on an AB Sciex 5800 MALDI-TOF-TOF with an Nd:YAG laser operating at 349 nm with a fixed beam diameter of 75 μm (Applied Biosystems, Foster City, CA, USA). Slides were placed in a microscope slide adaptor for the steel AB Sciex MALDI target (LaserBio Labs, Cat. # IMG-120) and affixed to the surface with copper tape that bridged the conductive surfaces of the MALDI plate and slide (Electron Microscopy Sciences, Hatfield, PA, USA, Cat. # 77801). For image acquisition, 4800 Imaging software (Applied Biosystems) was used to raster the microa rray surfaces collecting and averaging 50 spectra from points spaced 75 μM from center-to-center in *x* and *y* directions. Peptide analyses were performed in reflector positive mode scanning the m/z 500-2,500 range with a focus at m/z 1,250. For detection of m-YopH and NeutrAvidin, linear mid mass positive mode was used to scan the m/z 7,000-70,000 range with a focus m/z of 33,000. Monoclonal antibody (IgG) was detected in linear high mass positive mode scanning the m/z 100,000-200,000 range with a focus at m/z 150,000. See supplementary table S1 for additional details about MALDI settings.

Imaging data analysis

Optical images of the array area were exported from the Flexchip for coregistration with MS images. MS images were constructed from mass spectrometry data using TissueView software (Applied Biosystems). SPR data was collected and evaluated with Flex Imaging Software (GE Healthcare).

Results and Discussion

Direct evaluation of peptide microarrays by IMS

The first step in adapting microarray-based experiments to IMS was selecting a surface that was suitable for substrate immobilization and compatible with MALDI-TOF MS. We used ITO-coated glass slides as a printing medium based on conductive properties and chemical compatibility with immobilization methods [9]. In addition, these slides are inexpensive and widely available. To prepare the slides for analysis, a self-assembled monolayer of 11-MUA was applied to the ITO surface and NeutrAvidin was covalently linked to the SAM via EDC-sulfo-NHS. The biotinylated peptides were spotted onto the prepared slides and immobilized by high affinity biotin-NeutrAvidin interactions. The peptide microarrays were first evaluated by MALDI-IMS analysis, scanning a mass range encompassing the predicted peptide masses. A brief rinse in filtered, deionized water was used to remove residual salts and detergents deposited by the printing process and the surface was placed under a nitrogen gas stream for rapid and even drying before matrix application. To minimize sample delocalization and improve shot-to-shot reproducibility we used an automated sprayer to ensure even deposition and rapid drying of matrix solutions. In reflector mode, collecting 50 shots per spot at a 75 μm resolution required approximately 50 minutes for a 0.5 cm^2 microarray. As the AB5800 Nd-YAG laser has a fixed beam diameter of 75 μm, this configuration allowed for the maximum number of shots collected per microarray spot without oversampling (see Figure 1). The predominant peak for the each peptide was the sodium adduct, which was used to construct the MS images. Analyte distributions in these images reflected the grid pattern predicted from the programmed microarray printing, and these spots co-registered with an optical image of the microarray (Figure 2A-E and Table 1). Isobaric and nearly isobaric peptides appear in the same images. For example, [VDAAE(p)YLI^{+Na}] and [VAADE(p)YLI^{+Na}] are visible in an image constructed from m/z 1,333.47. Similarly, [AHX-VDADA(pY)LI^{+Na}] and [VDADEYLI$^{-H,\ +2Na}$] appear in the same image constructed from m/z 1,320.44. Lateral dispersion of the analyte is visible for some microarray spots. We speculate that this delocalization occurred during the initial wash step and not during matrix deposition, as the matrix is applied by the sprayer at a 90° angle

and the delocalization trends in a single direction (left-right) rather than radiating from the spot center.

IMS analysis of YopH catalytic activity

The MALDI-IMS results with the microarrayed peptides indicated that application of chemical matrix did not disrupt the printed ligands, suggesting that IMS was a practical alternative for data acquisition with microarray-based bioassays that require a high-throughput means of analysis. We next used MALDI-IMS to examine the enzymatic processing of microarrayed peptide substrates by YopH. Although *in vivo* protein targets of YopH are largely uncharacterized, a wide range of phosphotyrosine containing substrates are dephosphorylated *in vitro*, including phosphopeptides derived from the epidermal growth factor receptor (EGFR) [4; 5; 10]. We first examined a small microarray of EGFR phosphopeptides, employing the single catalytic domain (amino acid residues 164–468) of YopH to eliminate the domain-swapping binary complexes observed for the full-length protein [11]. The peptides selected for the library consisted of alanine substitutions and truncations of the native EGFR sequence derived from an EGFR autophosphorylation site (Tyr992). A central question in regulation of cell signaling is how phosphatases determine substrates for dephosphorylation among the numerous proteins encountered in the cell. Local sequence is considered to be one of the major factors and extensive efforts have been made to determine consensus sequences for dephosphorylation by specific phosphatases. Although EGFR is not known to be a target for dephosphorylation by YopH *in vivo*, previously conducted substrate activity screening have determined that the rate constant for YopH dephosphorylation of this sequence (2.23×10^7/M/second) is near the diffusion limit [4; 10; 12; 13].

The microarray surface was incubated with the active YopH, washed, and coated with matrix. The prepared slide was analyzed by MALDI-TOF-IMS, scanning the low m/z range encompassing the masses of the spotted peptides in reflector mode. Imaging of the microarray revealed phosphatase recognition of the phosphotyrosine-containing peptides, as indicated by reduced intensity of the phosphopeptide peak and the emergence of a peak corresponding to the dephosphorylated peptide mass. The efficiency of dephosphorylation was reproducible for each individual sequence but varied among the spotted peptides, as indicated by the relative pixel intensities of the phosphorylated and dephosphorylated versions of each peptide within spots of a wt-YopH treated microarray (Figure 2B-E). An estimate of catalytic efficiency can be derived for each peptide from the relative signal intensities of the mass peaks corresponding to the two peptides in the IMS spectra (Figure 2F).

These results suggested subtle preferences of the enzyme for select phosphotyrosine-containing substrate peptides. The importance of acidic residues at positions −1 and −2 and proline +3 from the phosphorylation site to catalytic activity has been noted previously [10]. The most efficiently dephosphorylated sequence in the microarray was indeed a 13-mer with Asp, Glu, and Pro at positions −1, −2, and +3, respectively. We noted that while phosphate removal from truncated versions of the EGFR peptide containing Ala substitutions was reduced compared to the full-length sequence, all phosphorylated peptides were dephosphorylated to some extent. Although the focus of our study was a bacterial protein tyrosine phosphatase, this strategy could conceivably be applied to other microarray-based enzymatic assays.

IMS analysis of YopH substrate affinity

Reasoning that protein adsorption to the microarray surface could also be visualized by MALDI-IMS with adjustments to matrix conditions and tuning parameters, we examined

phosphatase interactions with the phospho-peptide microarray. A duplicate microarray was incubated with m-YopH (the catalytically inactive mutant), washed, matrix-coated, and analyzed by MALDI-TOF-IMS. The microarray was scanned using linear mode to measure the high mass range encompassing the masses of YopH as well as the immobilized NeutrAvidin. The MS images constructed from this experiment confirmed that the substrate-trapping mutantof YopH bound specifically to the phosphotyrosine-containing peptides (Figure 3A). Again, the intensity of the peak corresponding to YopH was consistent among the triplicate peptide spots, and the overall intensity varied among the different peptide sequences. This particular result reinforced the critical role of phosphotyrosine in YopH substrate interactions, as no YopH binding (Figure 3) was detected in spots containing a non-phosphorylated sequence. In our case, IMS analysis of m-YopH binding to a peptide microarray highlighted the critical role of phosphotyrosine in YopH substrate affinity as well as a preference for acidic residues in the local sequence. It is likely that the wild-type enzyme catalyzes phosphate removal and immediately detaches from the dephosphorylated product, as previous kinetic studies with phosphotyrosine-containing peptide substrates demonstrated a weak association curve for the wt-YopH and a significantly stronger one for a catalytically inactive, substrate-trapping mutant of YopH [4; 14]. Taken together, these results suggested that the IMS-based phosphatase assay detected specific YopH binding and activity that was consistent with data collected by conventional kinetic and activity assays using a similar set of peptides [4; 10]. The total ion current and NeutrAvidin monomer images (Figures 3B and 3C, respectively) indicated a uniform reagent distribution across the imaged surface.

IMS coupled with surface plasmon resonance imaging of microarrays

On-chip MALDI-MS analysis was previously described as a method for augmenting data collected from SPR experiments, suggesting a logical extension to high-density SPRi microarrays [15; 16]. Although the conductive ITO-coated slides were optimal for MS studies, a thin gold or silver surface is required to detect surface plasmon resonance (SPR) resulting from molecular interactions. Therefore, we printed duplicate phosphopeptide microarrays on gold-coated polycarbonate chips for SPR analysis. An anti-phosphotyrosine monoclonal antibody was flowed over the EGFR peptide microarray and SPR responses were measured simultaneously for each of the twenty-seven microarray spots and an equal number of adjacent reference spots. Evaluation of the SPR response curves confirmed high-affinity binding only to the phosphotyrosine-containing peptides (Figure 4 A-I). After SPRi analysis, the chip was immediately retrieved and analyzed by MALDI-IMS using linear positive mode, scanning the high mass range associated with IgG antibodies (m/z 100,000-200,000). To visualize the presence of IgG, volume integration was performed on the area under the curve in the 100-200 kD range and converted to an optical image (Figure 4J). The resulting image indicated IgG binding specifically in the spots corresponding to phosphotyrosine peptides but not to non-phosphorylated peptides, in agreement with data obtained from the SPRi measurements.

In summary, we conclude that IMS analysis can be successfully coupled to microarray-based bioassays for an additional dimension of useful data. The imaging approach we describe simplifies mass spectrometric analysis of microarrays as it does not require alignment of the MALDI laser with individual microarray spots that are difficult to visualize after washes and matrix application. IMS also provides information about inter-spot areas that is useful for microarray quality control, and data acquisition can be coupled with SPR. Further, IMS analysis software is a convenient interface to view and compare spectra from specific microarray coordinates, allowing a detailed and interactive evaluation of the entire microarray surface. Combined with an unbiased IMS strategy, this enables multiplexed

detection of multiple analytes including non-targeted ones such as degraded peptides, contaminants, and novel enzymatic products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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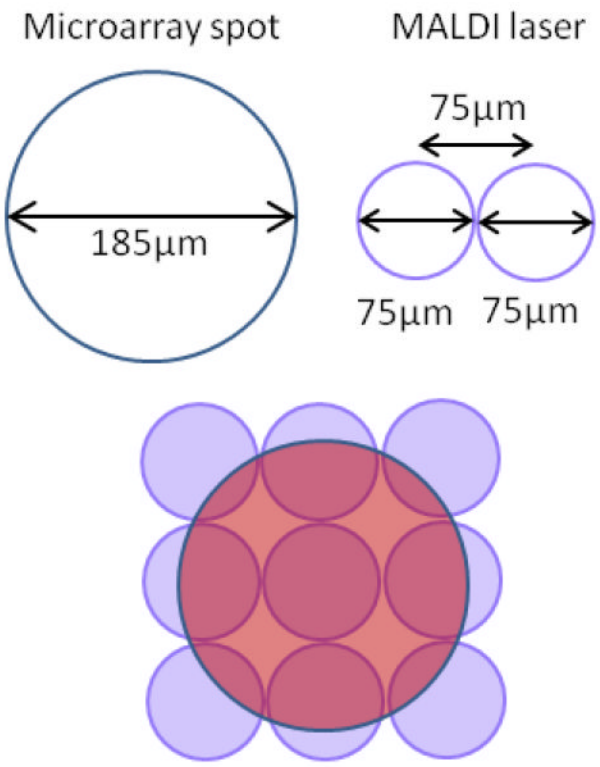

Figure 1.
MALDI laser raster overlayed on a 185 µm microarray spot. A 75 µm diameter Nd-YAG laser beam was used to generate ions from a raster of spots with 75 µm center-to-center spacing during automated MALDI-TOF data acquisition. This configuration collected the maximum number of samples per microarray spot without oversampling.

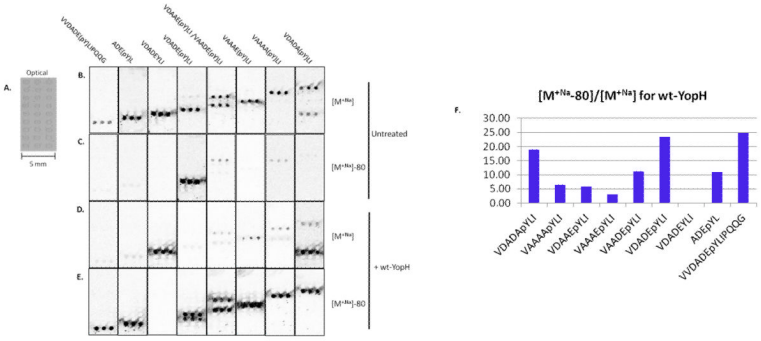

Figure 2.

MALDI-TOF imaging mass spectrometry of immobilized peptides. Nine peptides based on the sequence of a YopH target motif in EGFR were printed in triplicate in a 9 × 3 format. (A) Representative optical image of the microarray. (B) MS images depicting the intensity of peaks corresponding to the monoisotopic masses of the printed peptides. (C) Representative MS images depicting the intensity of peaks corresponding to the dephosphorylated versions of the printed peptides. (D and E) Representative MS images of the microarray after exposure to the wt-YopH in a phosphatase assay, monitoring the emergence of a $[M^{+Na}]$-80 peak corresponding to dephosphorylation of the peptide. Peptides that are isobaric or nearly isobaric appear, appear in the same images. (F) Relative intensity of peaks corresponding to dephosphorylated and phosphorylated peptides after exposure to wt-YopH. IMS spectra from the YopH phosphatase assay were exported and the peak intensities (centroid) corresponding to the $[M^{+Na}$-80] and $[M^{+Na}]$ mass for each peptide were compared. The $[M^{+Na}$-80] / $[M^{+Na}]$ value was used for a numerical comparison of the MS images for each peptide.

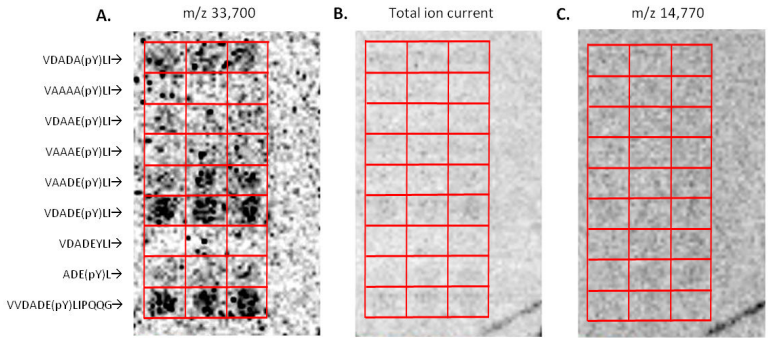

Figure 3.

MALDI-TOF imaging mass spectrometry of m-YopH bound to an EGFR peptide microarray. (A) MS image displaying the intensity (centroid, m/z 33,700) of a peak corresponding to m-YopH. (B) MS image of the scan range (7,000-70,000 m/z) total ion current. (C) MS image depicting the intensity of a peak corresponding to the immobilized NeutrAvidin monomer.

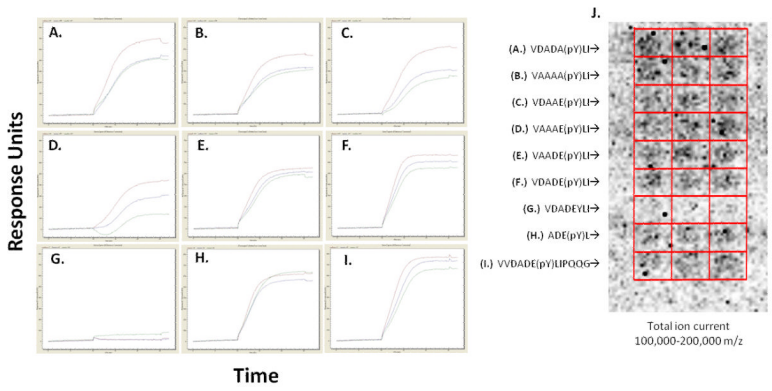

Figure 4.

Surface plasmon resonance imaging-coupled imaging mass spectrometry. (A-I) SPR sensorgrams of anti-phosphotyrosine antibody interactions with the triplicate peptide spots in each row. (J) MS image of the total ion current of the 100,000-200,000 m/z scan range from the retrieved SPRi chip.

Table 1

Variations of the EGFR-derived peptide “VDADEYLI” were synthesized with an N-terminal biotin and aminohexanoic acid (AHX) for immobilization and a C-terminal amide. The peptides were printed at equal concentrations across three columns of a 9 × 3 microarray.

Row	Columns	Peptide	M.W.
1	1-3	biotin-AHX-VDADA(pY)LI-amide	1297.44
2	1-3	biotin-AHX-VAAAA(pY)LI-amide	1209.42
3	1-3	biotin-AHX-VDAAE(pY)LI-amide	1311.47
4	1-3	biotin-AHX-VAAAE(pY)LI-amide	1267.46
5	1-3	biotin-AHX-VAADE(pY)LI-amide	1311.47
6	1-3	biotin-AHX-VDADE(pY)LI-amide	1355.48
7	1-3	biotin-AHX-VDADEYLI-amide	1275.48
8	1-3	biotin-AHX-ADE(pY)L-amide	1029.08
9	1-3	biotin-AHX-VVDADE(pY)LIPQQG-amide	1865.03